ORIGINAL ARTICLE

Progressive Changes of Colitis Into Colorectal Cancer Associated With Changes in Neutrophil Lymphocyte Ratio, Serum, Micrornas and Infiltrate Neutrophils

Pang Jyh Chyang[1], Nur Farhana Elida[1], Chin Siok Fang[2], Rahim Md Noah[1], Hanan Kumar[1]

[1] Institute of Medical Science Technology (MESTECH), Universiti Kuala Lumpur, 43000 Kajang Selangor, Malaysia
[2] Universiti Kebangsaan Malaysia, Institut Perubatan Molekul (UMBI), Malaysia

ABSTRACT

Introduction: Understand the progression of colorectal cancer from the beginning until the advance stages is difficult and challenging. However, this could be overcome with a good animal model. **Methods:** In this study, a modified approach had been used to develop colorectal cancer model. The model was developed and monitored from colitis formation until the late stage of colorectal cancer. The changes of neutrophil lymphocyte ratio (NLR), serum microRNAs and infiltrate neutrophil in different stages of colorectal cancer were assessed in this study. **Results:** Results showed that the progression of the disease is correlated with NLR as early as the formation of colitis ($r=0.121$, $p<0.026$). Meanwhile, the size of the tumor at each stage is also associated with the NLR value ($r=0.185$, $p<0.0012$). In the serum microRNAs study, it was found microRNAs expression in blood serum change in different stages of colorectal cancer. In the early stage of colitis formation, miR223 (> 3 fold expression, $p < 0.0025$) were abundantly found in the blood serum. Meanwhile in others stage mild (miRNA345 > 2.5 fold, $p<0.0011$), moderate (miRNA347 & miR512 > 3 fold, $p<0.002$) and severe (miR31 & miR145 > 2 fold, $p<0.0001$) microRNAs were also found expressed differently. The quantities of infiltrate neutrophil were varied in different stages of the disease. **Conclusion:** This study provides an insight into the immunity and molecular level of colorectal cancer and it allows a progressive monitoring on the changes in the molecular, cellular and histological level.
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Corresponding Author:
Pang Jyh Chyang, PhD
Email: jcpang@unikl.edu.my
Tel: +603 - 87395894

INTRODUCTION

Colorectal Cancer is the third most common diagnosed cancer with 1.8 million cases (1, 2). Recent global cancer data released by International Agency for Research on Cancer showed that colorectal cancer contribute 9.2% of total cancer mortality cases (IARC, WHO 2018). In Malaysia, colorectal cancer are the third common cancer and mostly diagnosed colorectal cancer cases are at the late stage and presented poor survival rate as compared to other cancer and the cancer case reported in other developing Asian countries (3, 4). Locally, the incidence of colorectal cancer is associated with some risk factors such as poor diet intake, low intensity of physical activities and smoking habits (5, 6). Commonly implemented treatment of colorectal cancer, surgical resection and immunotherapy, remains poor prognosis. This could be due to high recurrent rate of colorectal cancer and drug resistance effect.

Colorectal cancer is believed to be initiated with prolong inflammation activities in the colon due to inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease (7). Inflammation in the colon could trigger immunological responses (8). In this context, the neutrophil plays an important role in sustaining the inflammation activities. Furthermore, neutrophils have the highest population with up to 70% of the leukocytes in human blood (9, 10). Hence, understand the growth and progressive development of colorectal cancer and its association with neutrophil might be able to improve colorectal cancer therapeutic outcome. Therefore, in this study we are using the colitis model to imitate the colorectal cancer development and assess the progressive changes of the colorectal cancer model by using immunology, molecular and immunohistochemistry approach.

MATERIALS AND METHODS

In Vivo study (colitis-tumor model development)

A total of 30 male Wistar rats weighed average 250-300g was used in this study. The animals were acclimatized for a week in optimum laboratory condition. The rats were divided into four groups according Kilari et al.(11) method with a minor modification to induce different cancer severity. Colorectal cancer was induced by intraperitoneal injection of 16mg/kg of 1,2-Dimethylhydrazine and 0.01M Sulphuric acid, with 1.5% of EDTA as vehicle. The induction was carried out once per week for five consecutive weeks and incubated for ten weeks. The animals were sacrificed according to the incubation period of Kilari et al.(11).

Tissue block preparation

The tissues were inflated with 4% fresh paraformaldehyde and incubated for approximately two hours for fixation. The tissues were then placed and kept in phosphate buffer fixed at pH 7.4 with 30% sucrose and 20% OCT compound (Tissue-Tek) for 3 hours with intermittent inverting. After that the tissues were then removed from the solution and covered in OCT for 10 minutes. The colon tissues were kept frozen on dry ice, and stored at -80 oC.

Hematoxylin and Eosin staining

Tissue were sectioned, placed on slide and put on hot plate (ERMA INC. Hotplate (Tokyo, Japan) for 15 minutes. Slides were dewaxed with xylene (Merck Xylene) and gradually hydrated in absolute alcohol at concentrations of 100%, 90%, 80% and 70% each for 3 minutes. Slides were washed in running tap water for a minute and immersed in hematoxylin (Richard Allan Scientific Haematoxylin 2) for 5 minutes. After that the slides were washed under running water for 3 minutes, followed dipping these slides in clarifier (Richard Allan Scientific Clarifier) and were dipped twice in bluing agent (Richard Allan Scientific Bluing Agent). Lastly, the slides will be immersed in Eosin (Richard Allan Scientific Eosin) for two dips and dehydrated in two changes of 80% and 90% of absolute alcohol for 3 minutes each. Slides were placed in two changes of xylene (Merck Xylene) for 3 minutes and mounted with DPX (BDH DEPEX Mounting Medium), before covered with cover slip (TBS coverglass).

Blood serum microRNA

A total 200µL of serum were used to isolate microRNAs from the blood serum. The microRNAs were extracted according to miRNeasy kits (Qiagen, CA) protocol. The RNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE).

cDNA synthesis

Total cDNA was synthesis as described by promega GoScriptTM reverse transcription system protocol. Briefly, a total of 1µg of total RNA was incubate at 70°C for 10 minutes before added 4µl MgCl2, 2 µl reverse transcription buffer, 2 µl dNTP mixture, 0.5 µl recombinant RNasin, 15u AMV reverse transcriptase, 0.5 µg of OligodT primer and RNAse free water to make in a final volume of 20 µl.

Quantitative PCR

The quantitative PCR of the samples were done in 20µL total volume with 1µL of cDNA diluted 5 times, 10µL of 2X QuantiFast SYBR Green PCR master mix (Qiagen, Germany), and 250nM of each primer (Table 1). The cycling conditions of quantitative PCR were 95°C for 5 minutes followed by 35 cycles of 95°C for 10 seconds and 60°C for 30 seconds. While melting curve analysis was performed after the thermal profile.

Neutrophil lymphocyte ratio (NLR)

Blood count was done by using hemocytometer. The NLR was calculated as log e neutrophil count / log e lymphocyte count within the peripheral blood.

Immunohistochemistry study

Sections were incubated on a hot plate at 65°C for 30 minutes. An initial dewaxing step was done using 2 times xylene and rehydration step was performed using decreasing alcohol solutions with different concentrations of 100%, 80% and 70%. The slides was rinsed under running tap water for 3 minutes. The slides were subsequently incubated with hydrogen peroxide (Merck 30% Hydrogen Peroxide) at adjusted concentration followed by rinsed with running tap water. Antigen retrieval step was performed using heat –induced antigen retrieval pH 9.0 Tris-ethylenediaminetetraectic acid (EDTA)-based solution (DAKO) in the Pascal Pressurized Chamber (Dako Cytomation USA) with the conditions of temperature 97°C for 40 minutes followed by cooling at room temperature and was rinsed with running tap water. Slides were then incubated at room temperature with primary antibody then followed by incubation with secondary antibody (Dako RealTM Envision TM / HRP, Rabbit/Mouse Envision Kit) for 30 minutes. Sections were then incubated with 1 x DAB (Di- amino benzidine) –containing substrate working solution. Hematoxylins counter staining (Richard Allan Scientific Haematoxylin 2) was performed after the procedure had completed followed by dehydration in absolute alcohol and clearing in xylene. The completed slides were mounted using DPX mounting medium (BDH DEPEX Mounting Medium).

RESULTS

Histopathological analysis from colitis to colorectal cancer formation

The formation of colorectal cancer from colitis was analyzed using hematoxylin and eosin (Figure 1). The colitis and colorectal cancer was divided into three different stages (mild, moderate and severe) according to Kilari's study (11). In the initial stage of colitis formation,

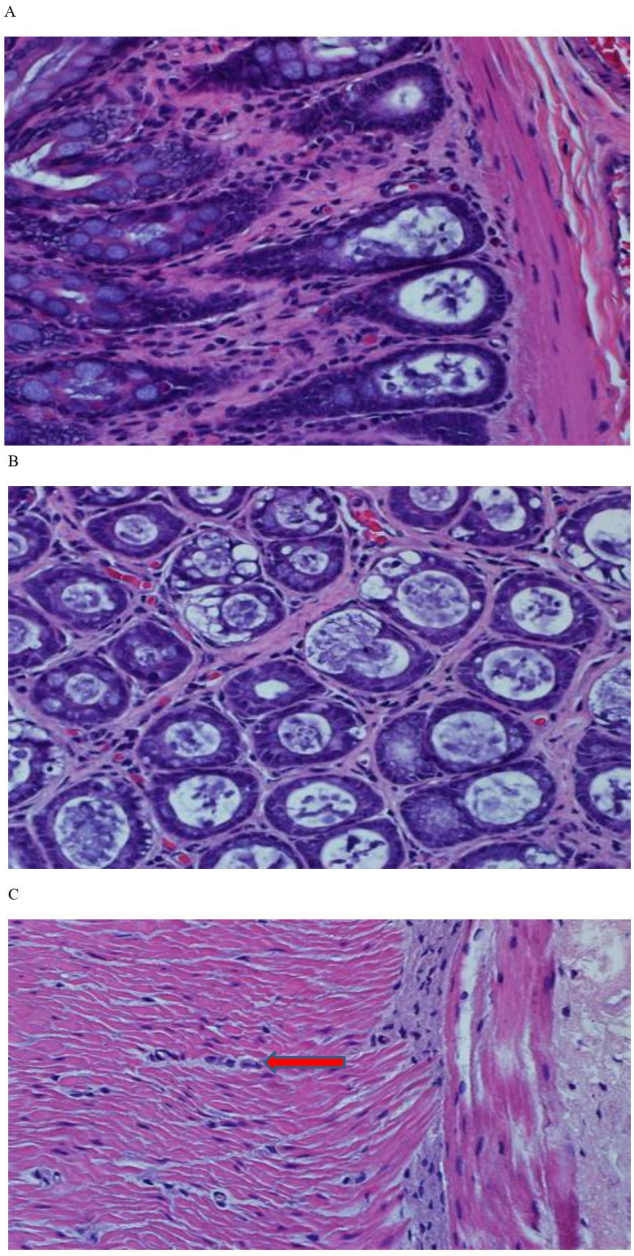

Figure 1: Histopathological analysis of colitis transforming into CRC via H&E staining. A –Mild stage of colitis: infiltration of immune cells, elongated and pseudostratified cells with reduced number of goblet cells. B – Moderate stage of colitis: Deformed nuclei with excessive cellular mitoses. C – Severe stage of colitis: The appearance of signet ring cells (arrow) and significant reduction of goblet cells. * Stage classification were referred from (12)

immune cells were found infiltrate into the area. The immune cells infiltrate into the colitis region were also observed in the moderate stage but not in the severe stage of colitis. Beginning from the moderate stage of colitis, the characteristics of cancer cell (deformed nucleic and extensive mitosis activities) have been observed.

In two months, colorectal cancer tissues were formed. In histopathological study (hematoxylin and eosin staining), immune cells were also found infiltrate into colorectal cancer tissue. Due to the formation of this colorectal cancer model was begin with colitis, therefore inflammatory activities were involved. Hence, neutrophil will be playing an important role in this transformation process. In the immunohistochemistry analysis, it was found that neutrophil changed its subset during the development of colorectal cancer (Figure 2). Meanwhile in the late stage of colorectal cancer, neutrophil was not found infiltrate into the tumor tissues.

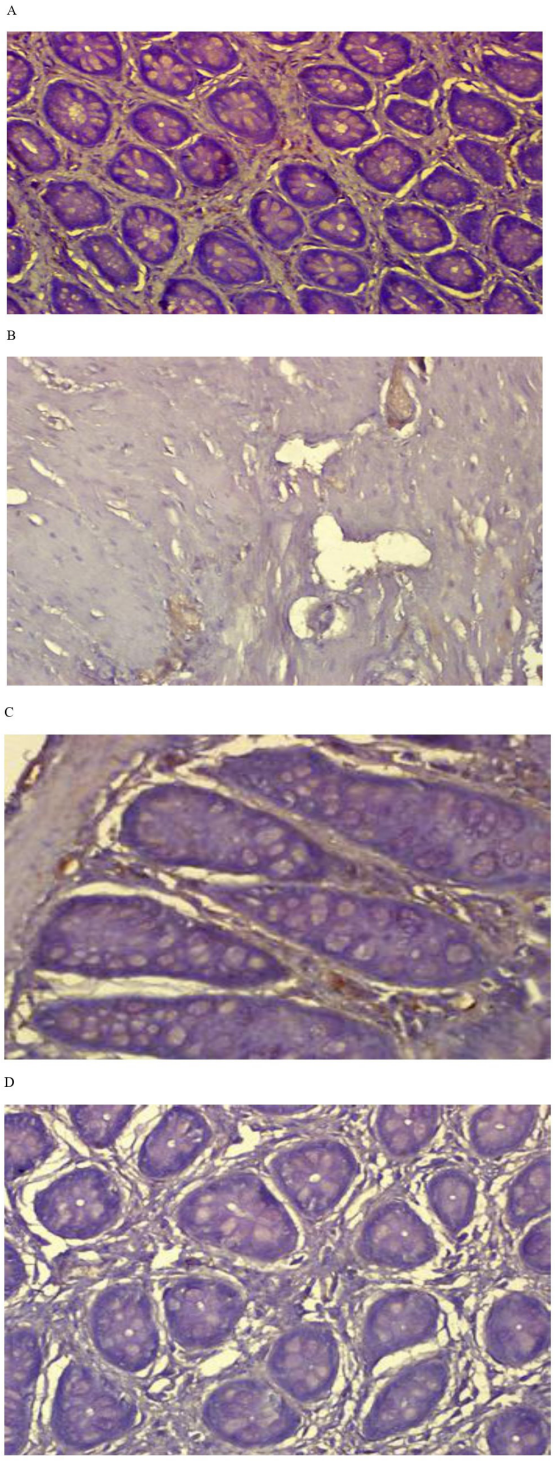

Figure 2: Histopathological analysis of colitis transformimg into CRC via IHC staining (colorectal tissue longitudinal section, 40X magnification). Infiltrating neutrophils were expressed as CD11. A – mild to moderate stage at colorectal mucosa. B – severe stage with poor cellular differentiation and CD11 poorly expressed. C – colorectal tissue at moderate stage with CD66b neutrophils subset infiltration. D – population of infiltrating neutrophils with CD66b expression was hardly observed at severe stage.

Neutrophil lymphocyte ratio

According to hallmarks of cancer, the development of colorectal cancer is associated with the interference of immunity cell function. In this study, we have shown that the changes of neutrophil lymphocyte ratio is associated with the different stages of colorectal cancer as well as the size of the tumor at the early stage of colorectal cancer. The neutrophil lymphocyte ratio was not increase significantly as compare with the mild stage and moderate stage of colorectal cancer (Figure 3).

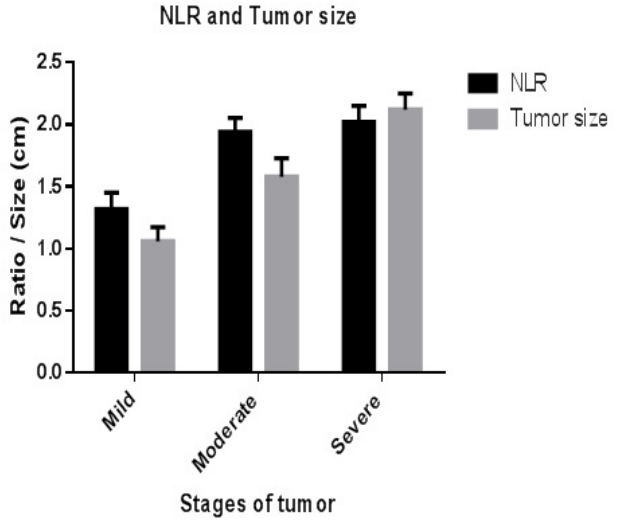

Figure 3: Neutrophil and leukocytes ratio (NLR) and tumor size in different stages of cancer

MicroRNA expression

The gene expression of the tumor cell is one of the crucial factors to ensure its sustainability to further develop. MicroRNA is one of the small sequence of RNA that controlling the gene expression activities. In this study six different microRNAs were choose (base on their biological function) to assess its expression in different stage of colorectal cancer. It was found that miR223 expression is significantly high in the early stage of colitis formation. Meanwhile, in the mild stage of colorectal cancer development miR345 expression is significantly high. At different stage of colorectal cancer development, microRNAs are different expressed (Figure 4).

DISCUSSION

Differential expression of serum microRNAs in different stage of tumor development was observedin this study. In mild stage miR345 is highly express. miR345 is the microRNA that targeting the expression of tumour suppressor gene, activities of peroxisome and ATP generation (13). In moderate stage, miR347 and miR512 is highly express in blood serum. Both miR347 and miR512 microRNAs are targeting on neuronal activities. Meanwhile in severe stage, highly expressed microRNAs, miR31 and miR145, are extensively involve in suppress cellular senescence and increase cellular

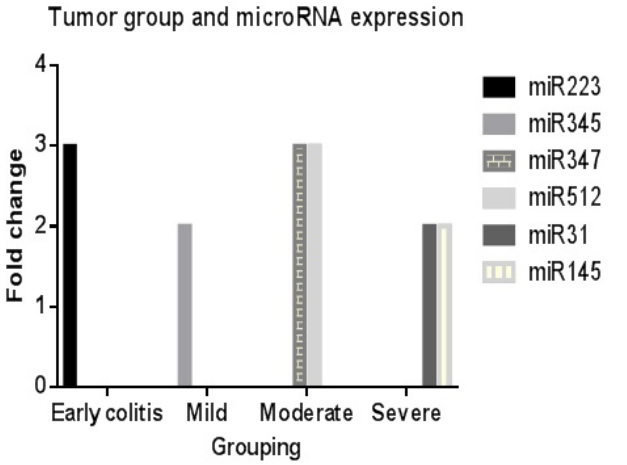

Figure 3: Differential microRNA expression in different stages of tumor

RNA polymerase activities.

In this study, the quantities of Neutrophil have changed in different stages of colorectal cancer. In order to interpret in more details on the changes of Neutrophil in different stages of colorectal cancer, Neutrophil lymphocyte ratio was used in this study. Neutrophil lymphocyte ratio was increased significantly in the early stage of colorectal cancer. The increment of neutrophil lymphocyte ratio is stagnant when disease advanced to severe stage (Figure 2). Neutrophil lymphocyte ratio analysis showed that neutrophil could be playing an important role in the early stage of tumor development. This result is also reported in Mustafa Goksu study (14) that Neutrophil lymphocyte ratio is increase together with tumor size of papillary thyroid.

Studies have shown that neutrophil has the possibility to polarize into different subtypes. In tumorigenesis process neutrophils change its plasticity role during tumor development (15). This phenomenon has been shown in this study as well. In the mild stage of colorectal cancer Neutrophil N1 (CD11) was found infiltrate into the colorectal tissues. However, when the colorectal cancer is progress into moderate stage, population of infiltrate neutrophil N1 (CD11) is replaced by neutrophil N2 (CD66b). Recently, neutrophil N2 has been found to have a role in release genotoxic DNA substances to increase DNA instability. In such, tumorigenesis process will be accelerated. However the mechanism that involve in transform neutrophil N1 to N2 in different stages of colorectal cancer is still unknown but it has been observed in this study.

The Neutrophil N1 and N2 were found infiltrate into the tissue at different stage of colorectal cancer. even though the quantities of Neutrophil is high in blood serum. Tumor might have utilized the microRNA expression to change the microenvironment and the immunity cell function of the host to evade from immunity attack.

CONCLUSION

Neutrophil did playing a roles in colorectal cancer development, begin from colitis formation until the moderate stage of colorectal cancer. However, the roles of Neutrophil in the late stage of colorectal cancer is still unclear as the quantities of Neutrophil in the serum of colorectal cancer model as well as its relation with differential microRNA expression is still unclear.

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